Generation of hydrogen peroxide precedes loss of mitochondrial membrane potential during DNA alkylation-induced apoptosis

Saeko Tada-Oikawa[a], Shinji Oikawa[a], Michiko Kawanishi[b], Michiyuki Yamada[c], Shosuke Kawanishi[a,*]

[a] *Department of Hygiene, Mie University School of Medicine, Edobashi, Tsu, Mie 514-8507, Japan*
[b] *Department of Microbiology, Faculty of Medicine, Kyoto University, Kyoto 606-8315, Japan*
[c] *Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanagawa-ku, Yokohama 236-0027, Japan*

Received 12 October 1998; received in revised form 27 November 1998

Abstract Pulsed field gel electrophoresis showed that the initiation time of DNA breakage induced by the DNA alkylating agent duocarmycin A, which is not a redox-cycling agent, was almost the same in the human leukemia cell line HL-60 and its H_2O_2-resistant clone HP100. Catalase activity of HP100 cells was much higher than that of HL-60 cells. Duocarmycin A-mediated DNA ladder formation in HP100 cells was delayed compared with that in HL-60 cells, suggesting the involvement of H_2O_2 in duocarmycin A-induced apoptosis. Flow cytometry demonstrated that peroxide formation preceded loss of mitochondrial membrane potential (ΔΨm) in cells treated with duocarmycin A. Then, caspase-3 was activated, followed by DNA ladder formation. These findings suggest that DNA damage by duocarmycin A induces H_2O_2 generation, which causes ΔΨm loss and subsequently caspase-3 activation, resulting in apoptosis.

Key words: Hydrogen peroxide; Mitochondrial membrane potential; Caspase-3; DNA damage; Apoptosis

1. Introduction

Reactive oxygen species (ROS) such as O_2^- and H_2O_2 are important regulators of apoptosis [1–3]. H_2O_2 can induce apoptosis [4] and antioxidants such as *N*-acetylcysteine [5], thioredoxin [6] and MnSOD [7] can delay or inhibit apoptosis. Other apoptotic stimuli, such as tumor necrosis factor α [8], ceramide [9] and lipopolysaccharide [10], result in generation of intracellular H_2O_2 during the apoptotic pathway.

It is well known that anticancer drugs, which cause DNA damage, can induce apoptosis [11–13]. Recent reports have suggested that DNA damage results in perturbation of inner mitochondrial membrane permeability [14–16]. Mitochondrial permeability transition (MPT), which involves a sudden increase of the mitochondrial membrane permeability, is a central coordination event of apoptosis [17–19]. It has been postulated that MPT could be caused by exogenous and endogenous ROS [17]. Although mitochondria are usually the main source of ROS in the cells, there remains the possibility that redox enzymes other than mitochondrial respiratory chain are sources of ROS. Here, we addressed the question of whether ROS generated from other systems than mitochondria activate MPT in the DNA damage-induced apoptotic pathway.

In this study, we investigated the causal relationship between DNA damage, generation of H_2O_2, and decrease in mitochondrial membrane potential (ΔΨm) during apoptosis. The ΔΨm disruption is mediated by opening MPT pores and appears to be critical for the apoptotic cascade [17]. We used duocarmycin A, an anticancer drug which induces DNA damage without H_2O_2 generation in a cell-free system, because duocarmycin A has highly specific DNA recognition and no redox-cycling activity [20,21]. That is, duocarmycin A alkylates specifically the adenine at A•T-rich sequences in double-stranded DNA [22] and induces cellular DNA damage [23]. HP100, which is a H_2O_2-resistant cell line derived from HL-60, was used to assess whether H_2O_2 participates in duocarmycin A-induced apoptosis. The catalase activity of HP100 cells was much higher than that of HL-60 cells [24]. In addition, since it is known that MPT initiates the activation of caspases [17,25,26], the time course of activation of caspase-1 (ICE) and caspase-3 (CPP32) was also investigated.

2. Materials and methods

2.1. Materials

Duocarmycin A was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Proteinase K was from Merck (Darmstadt, Germany). *z*-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) was from Enzyme System Products (Livermore, CA, USA). *z*-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) was from Biomol (Plymouth Meeting, PA, USA). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] were from Molecular Probes, Inc.

2.2. Cell culture and treatment with duocarmycin A

HP100 cells have been derived from HL-60 cells by repeated exposure to H_2O_2, followed by outgrowth of viable cells, and were approximately 340-fold more resistant to H_2O_2 than the parent cells, HL-60 [24]. Catalase activity of HP100 cells is 18 times higher than that of HL-60 cells. The HL-60 and HP100 cells were grown in RPMI 1640 supplemented with 6% FCS at 37°C under 5% CO_2 in a humidified atmosphere. Cells (10^6 cells/ml) were treated with 10 nM duocarmycin A throughout this experiment.

2.3. Detection of cellular DNA damage and DNA ladder formation induced by duocarmycin A

After treatment with duocarmycin A, the cells were washed with PBS. The cell suspension was solidified with agarose, followed by treatment with proteinase K according to the method as described previously [27]. Electrophoresis was performed by a CHEF-DRII pulsed field electrophoresis system (Bio-Rad) at 200 V at 14°C. Switch time was 60 s for 15 h followed by 90 s for 9 h. DNA ladder for-

*Corresponding author. Fax: (81) (59) 2315011.
E-mail: kawanisi@doc.medic.mie-u.ac.jp

Abbreviations: ROS, reactive oxygen species; ΔΨm, mitochondrial membrane potential; MPT, mitochondrial permeability transition; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DiOC6(3), 3,3′-dihexyloxacarbocyanine iodide; FACS, fluorescence-activated cell sorting; YVAD-AFC, *z*-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin; DEVD-AFC, *z*-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin

PII: S0014-5793(98)01618-4

mation, which is characteristic for apoptosis, was analyzed by conventional electrophoresis [28].

2.4. Flow cytometric detection of peroxide and ΔΨm

To evaluate cellular peroxide level, duocarmycin A-treated cells were incubated with 5 μM DCFH-DA for 30 min at 37°C [29]. To assess the change in ΔΨm, duocarmycin A-treated cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C [30]. Then, the cells were washed with PBS twice and suspended in PBS and then were analyzed with a flow cytometer (FACScan, Becton Dickinson). Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

2.5. Measurement of activities of caspase-1 and caspase-3

For analysis of activities of caspase-1 and caspase-3, the duocarmycin A-treated cells (10^6 cells) were washed with PBS twice and suspended in 100 μl of buffer A [0.1 M HEPES (pH 7.4), 2 mM DTT, 0.1% CHAPS, 1% sucrose]. The cell lysate was centrifuged at $18\,500 \times g$ for 15 min, and the supernatant was obtained. The reaction was initiated by addition of 20 μM YVAD-AFC or DEVD-AFC to 50 μl of apoptotic extract at 37°C and product formation was measured using a Hitachi F-2000 spectrofluorometer with excitation at 400 nm and emission at 505 nm.

3. Results

3.1. DNA damage and DNA ladder formation in HL-60 and HP100 cells treated with duocarmycin A

We have analyzed DNA strand breaks in the cells treated with duocarmycin A using pulsed field gel electrophoresis. DNA fragments corresponding to 1–2 Mb appeared not only in HL-60 cells but also in HP100 cells treated with duocarmycin A for 30 min (Fig. 1A), and then, 1–2-Mb fragments disappeared in HL-60 cells at 4 h, suggesting that the fragments were digested into internucleosomal DNA fragments.

In contrast, DNA ladder formation, which is characteristic for apoptosis, was already detectable at 2 h in HL-60 cells, but in the case of HP100 cells it was observed slightly at 3 h and apparently at 4 h (Fig. 1B). These time course data indicate that apoptosis mediated by DNA damage is delayed in HP100 cells, suggesting involvement of H_2O_2 in duocarmycin A-mediated apoptosis.

3.2. Generation of peroxide and change of ΔΨm in HL-60 and HP100 cells treated with duocarmycin A

To investigate whether generation of H_2O_2 precedes or follows the loss of ΔΨm, peroxide and ΔΨm in both HL-60 and HP100 cells treated with duocarmycin A were measured. As shown in Fig. 2, in HL-60 cells, peroxide generation was already observed at 30 min after treatment with duocarmycin A, whereas ΔΨm was drastically decreased at 2 h. On the other hand, in HP100 cells, peroxide generation occurred at 2 h, whereas ΔΨm was not altered at 2 h and apparently decreased at 3 h (Fig. 2). These results indicate that in HL-60 cells as well as in HP100 cells, duocarmycin A induces peroxide production followed by the loss of ΔΨm, although there is an about 1–1.5-h delay between the increase of peroxide level in HL-60 cells and that in HP100 cells.

3.3. Activation of caspase-3 by duocarmycin A

It has been well documented that caspase-1 and its related cysteine proteases such as caspase-3 play important roles in apoptosis [31–34]. Activation of caspase-1 and caspase-3 in duocarmycin A-treated cells was measured using a fluorometric assay with the substrates YVAD-AFC and DEVD-AFC,

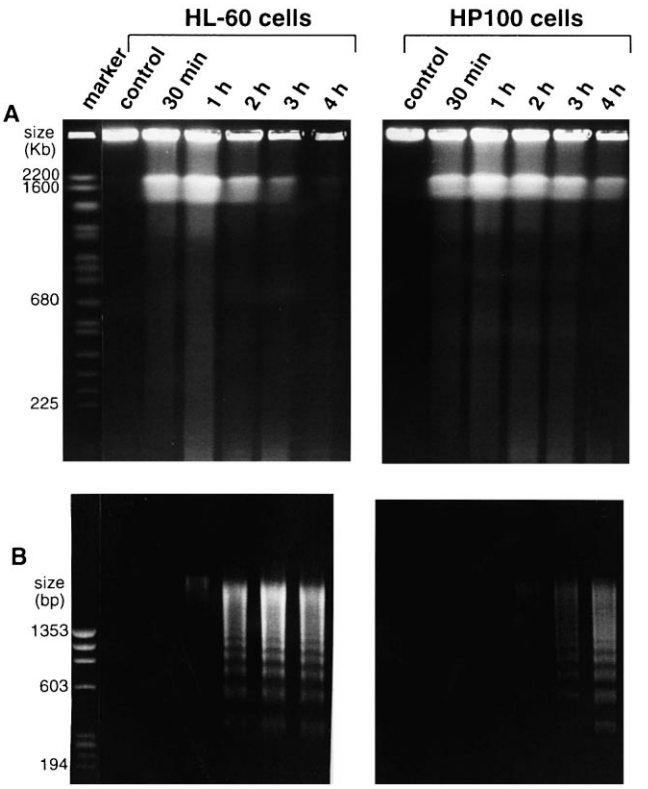

Fig. 1. Detection of cellular DNA damage and DNA ladder formation in HL-60 and HP100 cells treated with duocarmycin A. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with 10 nM duocarmycin A at 37°C for the indicated times. A: Cells were prepared as agarose plugs, lysed and subjected to pulsed field gel electrophoresis through a 1% agarose gel, as described in Section 2. The gel was stained in ethidium bromide. Marker lane: size maker DNA (*Saccharomyces cerevisiae*). B: The cells were lysed, and DNA was extracted and analyzed by conventional electrophoresis as described in Section 2. Marker lane: size marker DNA (ΦX174/*Hae*III digest).

respectively. In HL-60 cells, maximal caspase-3 activity was observed after treatment with duocarmycin A for 3 h (Fig. 3). In contrast, in HP100 cells, maximal caspase-3 activity was observed at 4 h after treatment with duocarmycin A (Fig. 3). There was a delay of about 1 h between the 50% of caspase-3 activity in HL-60 cells and that in HP100 cells. Caspase-1 was not activated by treatment with duocarmycin A in HL-60 cells (Fig. 3) and HP100 cells (data not shown). These results indicate that caspase-3 is involved in duocarmycin A-induced apoptosis and that DNA ladder formation follows the activation of caspase-3.

4. Discussion

The present study demonstrated that duocarmycin A induced DNA strand breakage in HL-60 and H_2O_2-resistant HP100 cells at the same time. However, subsequent events in the apoptotic pathway of HP100 cells were delayed compared with that of HL-60 cells. The catalase activity of HP100 cells was 18 times higher than that of HL-60 cells [24]. Therefore, it is considered that generation of H_2O_2 plays a critical role in duocarmycin A-mediated apoptosis but not in duocarmycin A-induced DNA damage.

Recent reports suggest that DNA damage results in onset of MPT, which plays a major role in the apoptotic process

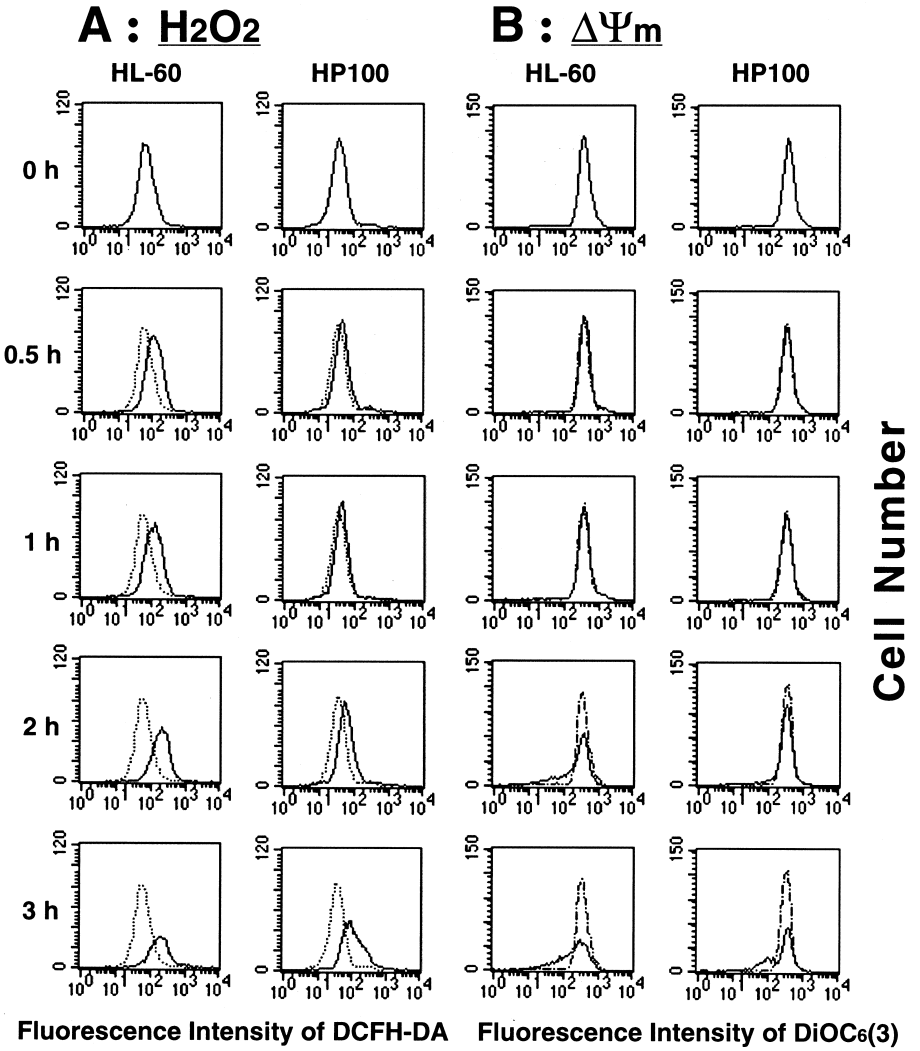

Fig. 2. Generation of H_2O_2 and the loss of ΔΨm in HL-60 and HP100 cells treated with duocarmycin A. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with 10 nM duocarmycin A at 37°C for the indicated times. After the treatment, the cells were incubated with 5 μM DCFH-DA for 30 min at 37°C (A) or 40 nM $DiOC_6(3)$ for 15 min at 37°C (B). The cells were analyzed with a flow cytometer (FACScan). The horizontal axis shows the relative fluorescence intensity, and the vertical axis shows the cell number. Dotted curves at 0.5–3 h correspond to control cells, like the solid curves at 0 h. Solid curves at 0.5–3 h represent duocarmycin A-treated cells.

[14–16]. A common step in apoptosis involves the loss of ΔΨm resulting in increased generation of ROS from the mitochondrial respiratory chain [17,25]. However, the mechanisms involved in the loss of ΔΨm caused by DNA damage remain to be clarified. The present study has shown that in HL-60 cells, the level of peroxide began to increase at 30 min after the treatment with duocarmycin A, and ΔΨm was decreased at about 1.5 h. In contrast, in HP100 cells, the level of peroxide began to increase at 1 h, and ΔΨm began to decrease at 2 h. Although DCFH-DA is used for detecting peroxide, it is considered on the basis of the comparison of HL-60 and HP100 cells that the peroxide detected in this experiment is mostly H_2O_2. These data are the first to demonstrate that H_2O_2 formation precedes the loss of ΔΨm in the DNA damage-induced apoptotic process. DNA damage and H_2O_2 production were likely to be parallel events triggered by duocarmycin A. However, since duocarmycin A is not a redox-cycling agent, we reasonably consider a possibility that H_2O_2 is produced by DNA damage induced by duocarmycin A. The resultant intracellular H_2O_2 produced by DNA damage might attack mitochondrial membranes. This idea is supported by a recent paper suggesting that O_2^- production activates MPT [2].

Here, the question has been raised how DNA damage induces O_2^- and subsequent H_2O_2 generation before the onset of MPT. There are several papers suggesting that mitochondria are the main source of ROS and that MPT contributes the generation of O_2^- and subsequent H_2O_2 generation [2,25,35]. However, mechanisms underlying ROS generation in other systems than mitochondria remain unclear. A recent study demonstrated rapid and specific H_2O_2 generation via NADH oxidase activation during Fas-mediated apoptosis [36]. In addition, Polyak et al. reported that p53, which can be induced by DNA damage, may result in apoptosis through the transcriptional induction of redox-related genes and the formation of ROS [37,38]. This possibility would be excluded in the present study since HL-60 is a p53-null cell line [39]. Alternatively, a mechanism for the DNA damage-induced ROS may be explained on the basis of the observation that DNA strand breakage can lead to activation of poly(ADP-

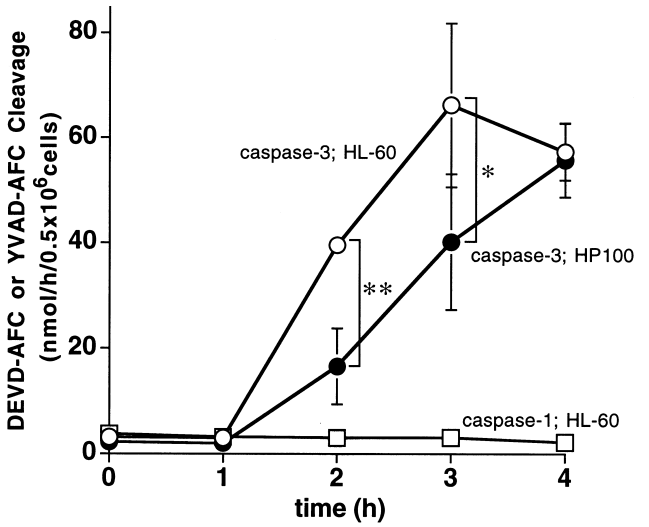

Fig. 3. Activation of caspase-1 and -3 in duocarmycin A-treated cells. Caspase-1 and -3 activities were measured by cleavage of YVAD- and DEVD-AFC respectively, using extracts of HL-60 and HP100 cells undergoing duocarmycin A-induced apoptosis. The cells (1×10^6 cells/ml) were treated with 10 nM duocarmycin A at 37°C for the indicated times. After the treatment, samples were assessed for YVAD- or DEVD-specific protease activation. Reactions were initiated by addition of 20 μM YVAD- or DEVD-AFC to the cell extract. Product formation (protease activity) was monitored by measuring the fluorescence (excitation at 400 nm, emission at 505 nm). ○, Activity of caspase-3 in HL-60; ●, activity of caspase-3 in HP100; □, activity of caspase-1 in HL-60. Results are means ± S.D. of values obtained from four independent experiments. Asterisks indicate significant differences between HL-60 cells and HP100 cells by *t*-test (**$P<0.01$, *$P<0.05$).

ribose) polymerase (PARP) with resulting loss of NAD^+ and ATP, which decreases the GSH/GSSG ratio [40,41]. Recent studies suggest that a depletion of GSH regulates MPT [42,43]. Therefore, it is reasonably considered that the intracellular GSH decrease via DNA damage results in an accumulation of excessive amounts of ROS and subsequently MPT may be activated, followed by cell death. Although an early increase in ROS is coupled to GSH depletion, a later increase in ROS may be additionally derived from the mitochondrial respiratory chain through the MPT pore affected by an early increase in ROS [1,14,19].

Previous studies have shown that cytochrome *c*, which is released from mitochondria during the induction of apoptosis, can initiate cleavage of pro-caspase-3 and activation of caspase-3 [26]. The present study suggests that the caspase-3 activation induced by duocarmycin A increases immediately after the loss of $\Delta\Psi m$. This led us to an idea that during duocarmycin A-induced apoptosis the loss of $\Delta\Psi m$ results in the activation of caspase-3. It was also reported that the activated caspase-3 cleaved DNA fragmentation factor, which is a human homologue of the mouse inhibitor of caspase-activated deoxyribonuclease [44,45], and led to DNA fragmentation at internucleosomal sites. Thus, it is concluded that DNA damage induces the generation of H_2O_2 which causes MPT, and subsequently initiates the activation of caspase-3, resulting in apoptosis.

Acknowledgements: This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] Tan, S., Sagata, Y., Liu, Y., Maher, P. and Schubert, D. (1998) J. Cell Biol. 141, 1423–1432.
[2] Cai, J. and Jones, D.P. (1998) J. Biol. Chem. 273, 11401–11404.
[3] Banki, K., Hutter, E., Gonchoroff, N.J. and Perl, A. (1998) J. Biol. Chem. 273, 11944–11953.
[4] Gardner, A.M., Xu, F.H., Fady, C., Jacoby, F.J., Duffey, D.C., Tu, Y. and Lichtenstein, A. (1997) Free Radical Biol. Med. 22, 73–83.
[5] Mayer, M. and Noble, M. (1994) Proc. Natl. Acad. Sci. USA 91, 7496–7500.
[6] Iwata, S., Hori, T., Sato, N., Hirota, K., Sasada, T., Mitsui, A., Hirakawa, T. and Yodoi, J. (1997) J. Immunol. 158, 3108–3117.
[7] Majima, H.J., Oberley, T.D., Furukawa, K., Mattson, M.P., Yen, H.C., Szweda, L.I. and St. Clair, D.K. (1998) J. Biol. Chem. 273, 8217–8224.
[8] Mannna, S.K., Zhang, H.J., Yan, T., Oberley, L.W. and Aggarwal, B.B. (1998) J. Biol. Chem. 273, 13245–13254.
[9] Quillet-Mary, A., Jaffrézou, J.-P., Mansat, V., Bordier, C., Naval, J. and Laurent, G. (1997) J. Biol. Chem. 272, 21388–21395.
[10] Wang, J.H., Redmond, H.P., Watson, R.W. and Bouchier-Hayes, D. (1997) Am. J. Physiol. 272, C1543–1551.
[11] Thompson, C.B. (1995) Science 267, 1456–1462.
[12] Müller, I., Jenner, A., Bruchelt, G., Niethammer, D. and Halliwell, B. (1997) Biochem. Biophys. Res. Commun. 230, 254–257.
[13] Hickman, J.A. (1992) Cancer Metast. Rev. 11, 121–139.
[14] Mignotte, B. and Vayssiere, J.-L. (1998) Eur. J. Biochem. 252, 1–15.
[15] Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z.-M., Saxena, S., Weichselbaum, R., Nalin, C. and Kufe, D. (1997) Proc. Natl. Acad. Sci. USA 94, 6939–6942.
[16] Tada-Oikawa, S., Oikawa, S. and Kawanishi, S. (1998) Biochem. Biophys. Res. Commun. 247, 693–696.
[17] Kroemer, G., Zazami, N. and Susin, S.A. (1997) Immunol. Today 18, 44–51.
[18] Green, D.R. and Reed, J.C. (1998) Science 281, 1309–1312.
[19] Kowaltowski, A.J., Netto, L.E.S. and Vercesi, A.E. (1998) J. Biol. Chem. 273, 12766–12769.
[20] Takahashi, I., Takahashi, K., Ichimura, M., Morimoto, M., Asano, K., Kawamoto, I., Tomita, F. and Nakano, H. (1988) J. Antibiot. 41, 1915–1917.
[21] Gomi, K., Kobayashi, E., Miyoshi, K., Ashizawa, T., Okamoto, A., Ogawa, T., Katsumata, S., Mihara, A., Okabe, M. and Hirata, T. (1992) Jpn. J. Cancer Res. 83, 113–120.
[22] Yamamoto, K., Sugiyama, H. and Kawanishi, S. (1993) Biochemistry 32, 1059–1066.
[23] Okamoto, A., Okabe, M. and Gomi, K. (1993) Jpn. J. Cancer Res. 84, 93–98.
[24] Kasugai, I. and Yamada, M. (1992) Leukemia Res. 16, 173–179.
[25] Kim, C.N., Wang, X., Huang, Y., Ibrado, A.M., Liu, L., Fang, G. and Bhalla, K. (1997) Cancer Res. 57, 3115–3120.
[26] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[27] Ito, K., Yamamoto, K. and Kawanishi, S. (1992) Biochemistry 31, 11606–11613.
[28] Hiraku, Y. and Kawanishi, S. (1996) Cancer Res. 56, 5172–5178.
[29] Xiang, J., Chao, D.T. and Korsmeyer, S.J. (1996) Proc. Natl. Acad. Sci. USA 93, 14559–14563.
[30] Decaudin, D., Geley, S., Hirsch, T., Castedo, M., Marchetti, P., Macho, A., Kofler, R. and Kroemer, G. (1997) Cancer Res. 57, 62–67.
[31] Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) Cell 87, 171.
[32] Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) J. Biol. Chem. 269, 30761–30764.
[33] Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell 90, 405–413.
[34] Thornberry, N.A. and Lazebnik, Y. (1998) Science 281, 1312–1316.
[35] Duranteau, J., Chandel, N.S., Kulisz, A., Shao, Z. and Schumacker, P.T. (1998) J. Biol. Chem. 273, 11619–11624.

[36] Suzuki, Y., Ono, Y. and Hirabayashi, Y. (1998) FEBS Lett. 425, 209–212.
[37] Polyak, K., Xia, Y., Zweider, J.L., Kinzler, K.W. and Vogelstein, B. (1997) Nature 389, 300–305.
[38] Johnson, T.M., Yu, Z.X., Ferrans, V.J., Lowenstein, R.A. and Finkel, T. (1996) Proc. Natl. Acad. Sci. USA 93, 11848–11852.
[39] Dou, Q.P., An, B. and Will, P.L. (1995) Proc. Natl. Acad. Sci. USA 92, 9019–9023.
[40] Marini, M., Frabetti, F., Brunelli, M.A. and Raggi, M.A. (1993) Biochem. Pharmacol. 46, 2139–2144.
[41] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, 2nd edn., Oxford University Press, Oxford.
[42] Backway, K.L., McCulloch, E.A., Chow, S. and Hedley, D.W. (1997) Cancer Res. 57, 2446–2451.
[43] Costantiti, P., Chernyak, B.V., Petronilli, V. and Bernardi, P. (1996) J. Biol. Chem. 271, 6746–6751.
[44] Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) Cell 89, 175–184.
[45] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) Nature 391, 43–50.